

FROM THE DEPARTMENT OF HISTOLOGY,
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THE GLYOXYLIC ACID FLUORESCENCE
HISTOCHEMICAL METHOD FOR MONOAMINES

CHEMISTRY, METHODOLOGY AND NEUROANATOMICAL APPLICATION

BY

OLLE LINDVALL

LUND 1974

From the Department of Histology, University of Lund,
Lund, Sweden

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To my parents, Marianne and Gustaf Lindvall

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The present symposium is held on the following papers:

- I Fluorescence spectroscopy of biological membranes: A study of the mobility of various colored compounds to test fluorescence of the probe, dependence on glycerol content. J. Björklund, *Cytochrome*, 20, 407-426, 1973 (together with S. Axelsson and A. Ernster)
- II Mechanism of the glucose formation in the cytoplasm of the liver and method for measurement. *Thrombosis & Haemostasis*, 32, 1-12, 1974 (together with S. Björklund and L.A. Sjöström)
- III New aspects of membrane metabolism in the liver and method for measurement. J. Björklund, *Cytochrome*, 20, 427-438, 1973 (together with A. Björklund, S. Teller and L.A. Sjöström)
- IV Elementary reactions in the cytoplasm and related compounds in the cytoplasm and fluorescence spectroscopy method. *Thrombosis & Haemostasis*, 32, 135-139, 1974 (together with S. Teller and L.A. Sjöström)
- V Fluorescence spectroscopy of lipids: A study of the effect of COOH- and NH₂-groups on the fluorescence of lipids by condensation with glyoxylic acid. J. Björklund, *Cytochrome*, 21, 255-265, 1974 (together with S. Björklund, K. Nilsson and F. Sjöström)
- VI The glyoxylic acid fluorescence method: A detailed account of the methodology for the visualization of central catecholamine neurons. *Neurochemistry*, 30, 97-127, 1974 (together with A. Björklund)
- VII The organization of the ascending catecholamine neuron system in the rat brain as revealed by the glyoxylic acid fluorescence method. *Ann. N.Y. Acad. Sci., suppl.*, 212, 1075 (together with S. Björklund)
- VIII The descending catecholamine neuron system as revealed by the glyoxylic acid fluorescence method. J. Comp. Neurol., 198, 317-345, 1974 (together with A. Björklund, S. Teller and L.A. Sjöström)

To my parents, Marianne and Gustaf Lindvall

These papers will be referred to by their Roman numerals.

The present summary is based on the following papers:

- I Fluorescence histochemistry of biogenic monoamines. A study of the capacity of various carbonyl compounds to form fluorophores with biogenic monoamines in gas phase reactions. *J. Histochem. Cytochem.* 20, 435-444, 1972 (together with S. Axelsson and A. Björklund)
- II Mechanisms of fluorophore formation in the histochemical glyoxylic acid method for monoamines. *Histochemie* 32, 113-131, 1972 (together with A. Björklund and L.Å. Svensson)
- III New aspects on reaction mechanisms in the formaldehyde histofluorescence method for monoamines. *J. Histochem. Cytochem.* 21, 17-25, 1973 (together with A. Björklund, B. Falck and L.Å. Svensson)
- IV Fluorophore formation from catecholamines and related compounds in the glyoxylic acid fluorescence histochemical method. *Histochemistry* 39, 197-227, 1974 (together with A. Björklund and L.Å. Svensson)
- V Fluorescence histochemical demonstration of peptides with NH_2 - or COOH -terminal tryptophan or dopa by condensation with glyoxylic acid. *J. Histochem. Cytochem.* 21, 253-265, 1973 (together with A. Björklund, R. Håkanson and F. Sundler)
- VI The glyoxylic acid fluorescence histochemical method: a detailed account of the methodology for the visualization of central catecholamine neurons. *Histochemistry* 39, 97-127, 1974 (together with A. Björklund)
- VII The organization of the ascending catecholamine neuron systems in the rat brain as revealed by the glyoxylic acid fluorescence method. *Acta physiol. scand. suppl.* 412, 1974 (together with A. Björklund)
- VIII The adrenergic innervation of the rat thalamus as revealed by the glyoxylic acid fluorescence method. *J. Comp. Neurol.* 154, 317-348, 1974 (together with A. Björklund, A. Nobin and U. Stenevi)

These papers will be referred to by their Roman numerals.

I INTRODUCTION

The fluorescence histochemistry represents an extremely sensitive methodological approach to qualitative and quantitative studies of cellular amine stores in neuronal as well as non-neuronal tissues. The methods based on condensation with gaseous formaldehyde (FA) are the most sensitive and the most widely used ones, and in its classical form as devised by Falck, Hillarp and coworkers (Falck, 1962; Falck *et al.*, 1962; Corrodi and Hillarp, 1963, 1964) the FA technique permits the visualization of very low amounts of primary catecholamines (CAs) and 5-hydroxytryptamine, *e.g.* in their intraneuronal storage sites. The sensitivity of the Falck-Hillarp method can be expressed as the lowest amount of substance detectable in a nerve terminal varicosity. The amount of noradrenaline (NA) present in one varicosity of the sympathetic nerves of the rat iris (having a diameter of approximately $1\ \mu$) has been calculated to be about 5×10^{-3} pg (Dahlström *et al.*, 1966). From the observations of Jonsson (1969) that such varicosities containing less than 10% of their normal NA content can be visualized, it appears that as little as 5×10^{-4} pg (about 5×10^{-6} picomoles) of NA can be readily detected within one varicosity.

Further developments of the FA method, the combined FA-ozone reaction (Björklund *et al.*, 1968 b) and the acid-catalyzed FA reaction (Björklund and Stenevi, 1970; Björklund *et al.*, 1971) have made possible highly sensitive histochemical demonstration also of tryptamine, NH_2 -terminal tryptophanyl peptides (Håkanson and Sundler, 1971) and 3-methoxylated β -phenylethylamines. Also, the recently introduced Vibratome sectioning technique (Hökfelt and Ljungdahl, 1972) involves an increased sensitivity for brain CAs in the Falck-Hillarp method in addition to an improved morphology of the tissue sections. Apart from these FA methods, fluorescence histochemical techniques have been presented for the demonstration of adrenaline – by oxidation with gaseous iodine to trihydroxyindole derivatives (Carlsson *et al.*, 1961; Angelakos and King, 1967) – and of histamine, by reaction with *o*-phthalaldehyde (Juhlin and Shelley, 1966; Ehinger and Thunberg, 1967; Håkanson and Owman, 1967). In their present shape, both these methods have a lower sensitivity than the FA techniques and do not allow, *e.g.*, the demonstration of adrenaline and histamine in mammalian nervous tissues.

Available fluorescence histochemical methods have obvious limitations, and there is a strong demand for new supplementary techniques. Thus, there is a need for new

fluorescence histochemical methods both for a more sensitive visualization of amines now demonstrable with the FA method, e.g., dopamine (DA), NA and 5-hydroxytryptamine, and for demonstration of amines which are known to occur in mammalian tissues, but cannot be visualized or cannot be visualized with sufficient sensitivity with existing methods. Examples of such biologically interesting amines are melatonin, tyramine, octopamine, and histamine.

In the present investigation the histochemical usefulness of a number of reactive carbonyl compounds was studied and the most promising one, glyoxylic acid (GA), was applied as a fluorescence histochemical reagent for biogenic monoamines. The aim of the study was to devise a useful fluorescent histochemical method based on this reagent. Thus, the methodology for different types of tissue, the chemical background to the method, and the possibilities for microspectrofluorometric identification of and differentiation between various GA-induced fluorophores were investigated. The usefulness of the GA method for neuroanatomical studies on central CA neurons was evaluated in particular, and with the GA technique the organization of the ascending CA neurons and the adrenergic innervation of the thalamus in the rat brain were studied in detail.

II CHEMISTRY

a. Fluorophore formation from biogenic amines in reactions with carbonyl compounds (Paper I)

In order to find potentially useful reagents for fluorescence histochemical demonstration of biogenic monoamines 20 different compounds, having a carbonyl group ($>C=O$) as a common characteristic, were tested for their capacity to form fluorophores with these substances under histochemical gas phase reaction conditions (see Paper I). The low molecular aldehydes were found to yield the strongest fluorescence with the catecholamines, DA and NA, and the indolamines, tryptamine and 5-hydroxytryptamine. The most reactive carboxylic acids, α -keto acids and ketones gave quite distinct but weaker fluorescence. When considering the reaction selectivity, which is of primary importance for the histochemical usefulness of a reagent, it was found that under the present reaction conditions only a few reagents fulfilled these requirements: FA, heptaldehyde, chloral, GA, acetic acid (cf. Rost and Ewen, 1971), pyruvic acid and acetylchloride. Of these, only FA and GA gave a strong fluorescence, thus combining high reactivity with good selectivity.

Distinct fluorescence from the N-acetylated indolamines, melatonin and N-acetyl-5-hydroxytryptamine, was obtained with four acids, GA, formic acid, acetic acid, and pyruvic acid. Heptaldehyde and formic acid gave a good visible (although quite weak) fluorescence in the reaction with histamine, and chloral and formic acid induced a low fluorescence from p-tyramine.

Of the reagents with interesting properties, GA appeared to be the most promising one for amine histochemistry. In a preliminary report (Axelsson, Björklund, Falck, Lindvall and Svensson, 1973) it was shown that GA could demonstrate intracellular monoamines, and that this reagent was more sensitive than FA for the well-known CAs and indolamines and that it also should allow the detection of other biogenic amines — such as melatonin — not demonstrable with available techniques.

In addition to GA, which has been developed further as a fluorescence histochemical reagent (Papers II and IV-VIII), pyruvic acid and acetic acid have been successfully applied to tissue (unpublished observations). It should be of considerable

interest to investigate the histochemical usefulness and the mechanisms of fluorophore formation of these and other interesting reagents in detail, particularly those forming fluorophores with N-acetylated indolamines, histamine and p-tyramine, substances that cannot be demonstrated with sufficient sensitivity with the FA techniques. This seems to be of particular value also because the mechanisms of fluorophore formation in several cases represent entirely new fluorescence histochemical reactions.

b. Fluorophore formation from biogenic amines in the glyoxylic acid histochemical reaction (Papers II and IV)

Reaction between phenylethylamines and glyoxylic acid (Paper IV). The mechanisms of fluorophore formation, which were analyzed in detail for DA, involve two main steps analogous to the FA reaction (Corrodi and Hillarp, 1963, 1964; Corrodi and Jonsson, 1967): First a Pictet-Spengler type cyclization to the weakly fluorescent 1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid; second, by an intramolecularly catalyzed reaction of the tetrahydro derivative with a second GA molecule, yielding the strongly fluorescent 2-carboxymethyl-3,4-dihydroisoquinolinium compound.

The fluorophore yield (i.e. the yield of the strongly fluorescent 2-carboxymethyl derivative) from DA in the GA vapour reaction (studied with ^{14}C -labelled DA enclosed in histochemical protein models) was found to be about 10%, the tetrahydroisoquinoline derivative constituting the main reaction product, about 80%. When the GA reaction was performed under conditions simulating the perfusion-immersion procedure used on tissue (see below), the yield of the fluorophore from DA was strongly enhanced, under these conditions being almost quantitative. This pronounced increase in the fluorophore yield can be referred to several phenomena (see Discussion in Paper IV): a) The increased concentration of GA at the reaction site; b) The efficient drying procedure that will change the reaction milieu in a way that probably favours the second step of the reaction (cf. Svensson, Björklund and Lindvall, 1974); c) The more vigorous reaction conditions employed.

It was remarkable that despite the yield of strongly fluorescent molecules being only about 10%, the fluorescence yield from DA in the GA vapour reaction was roughly the same as that obtained from equimolar concentrations of the synthetic 2-carboxymethyl derivative. A similar discrepancy between fluorophore and fluorescence yield was also observed in the GA-tryptamine reaction (Paper II). Several explanations can be given to this phenomenon (see Discussion in Paper IV): a) The quantum efficiency of the synthetic fluorophore could be lowered due to the presence of a quencher in the histochemical models; b) The fluorescence of the fluorophore molecules formed in the

histochemical reaction is enhanced due to interaction with other molecules. This could be in the form of an energy transfer between e.g. the tetrahydro and the 2-carboxymethyl derivatives, or via a binding of the fluorophore to the matrix forming a complex with higher quantum efficiency. It is evident that such a fluorescence enhancing mechanism in the gaseous GA and FA reactions should be of basic importance for the sensitivity of the techniques and of considerable interest, e.g., in the microfluorometric quantitation of amines at the cellular level.

Several factors proved to be of importance in determining the fluorescence yields from phenylethylamines (PEAs) in the GA reaction (cf. Methodology) e.g. the presence, number and structure of substituents on the PEA molecule, the reaction time, and the amount of GA present during the reaction. It was found that gaseous GA induced strong fluorescence only from 3-hydroxylated and certain 3-methoxylated, primary PEAs, the unsubstituted PEAs and those with hydroxyl and methoxyl groups in 2 or 4 position being essentially non-fluorescent. This is in agreement with the well-known fact (Whaley and Govindachari, 1951; Björklund and Stenevi, 1970) that, when performed under mild reaction conditions, the Pictet-Spengler cyclization is favoured by substituents in the aromatic ring giving a high electron density at the point of ring closure (the 6-carbon). This is fulfilled in PEAs having a hydroxyl or methoxyl group para to the position of closure, i.e. in the 3-position.

In the GA reaction strong fluorescence was obtained from several 3-methoxylated PEAs, e.g. 3-methoxytyramine and DMPEA, substances that give a low fluorescence intensity in the standard FA reaction. It is most probable that GA, being an acid, catalyzes its own cyclization with these compounds, which are known to be less reactive in the Pictet-Spengler reaction than those with a hydroxyl group in 3-position (Kovács and Fodor, 1951; Whaley and Govindachari, 1951; Björklund and Stenevi, 1970). The difference in reactivity between 3-hydroxylated and 3-methoxylated PEAs was observed also in the GA reaction. Thus, the fluorescence yields from PEAs with a methoxyl group in 3-position were lower than those from the corresponding 3-hydroxylated PEAs, and they were also more dependent on reaction time and on the GA concentration in the reaction vessel. These differences in reactivity indicates interesting possibilities for differentiation between the two types of amines by changing the reaction conditions in a proper way.

Interestingly, it was found that the presence, structure and number of substituents on the benzene ring and on the side-chain of the PEA molecule had characteristic effects on the fluorescence yields in the GA vapour reaction from the 3-hydroxylated and 3-methoxylated PEAs (for details, see paper IV). This should be

of considerable value in cytofluorometric analysis where fluorescence yields and spectral characteristics are used for the characterization and identification of intracellular monoamines (cf. Methodology).

The very low fluorescence yields from secondary PEAs in the GA reaction are most likely due to that the tetrahydroisoquinolines formed from these compounds cannot react with a second GA molecule, and therefore the efficient formation of the 2-carboxymethyl derivatives cannot take place. However, when prolonging the reaction time, the fluorescence yields from the secondary CAs increase markedly, probably due to autoxidation of the tetrahydro derivatives to strongly fluorescent dihydroisoquinolines.

Tertiary and N-acetylated CAs give, in contrast to the corresponding indolamines (Papers I, II, and VI) very low or no fluorescence upon GA treatment. These compounds cannot enter a Pictet-Spengler cyclization and consequently the fluorophore formation must proceed differently (cf. below). However, for the detection of N-acetylated and tertiary CAs in tissue this histochemical reaction seems to be of no practical value.

Reaction between indolyethylamines and glyoxylic acid. The mechanisms of fluorophore formation, which were analyzed in detail for tryptamine (Paper II), were shown to be similar to those in the reaction between GA and PEAs (see above). Thus, the GA-tryptamine reaction proceeds through an initial Pictet-Spengler type cyclization to the 1,2,3,4-tetrahydro- β -carboline-1-carboxylic acid, followed by two alternative fluorophore forming reactions yielding the 3,4-dihydro- β -carboline, or the 2-carboxymethyl-3,4-dihydro- β -carbolinium (main fluorophore) and 2-methyl-3,4-dihydro- β -carbolinium compounds, which are all strongly fluorescent.

Most probably, other primary indolamines and the NH_2 -terminal tryptophan peptides (Paper V) react in a similar way. N-acetylated and tertiary indolamines and COOH-terminal tryptophan peptides, which give a strong fluorescence in the GA reaction, do not have the molecular requirements for a Pictet-Spengler cyclization reaction. Consequently, the fluorophore formation, which has not yet been elucidated, must proceed differently. One possible mechanism of fluorophore formation from N-acetylated indolamines and COOH-terminal tryptophan peptides is via a so-called Bischler-Napieralski reaction (see Papers I and V), i.e. a GA-promoted cyclodehydration to strongly fluorescent dihydro- β -carbolines.

The fluorophore yield (as studied with ^{14}C -labelled tryptamine; Paper II) was found to be low also in the GA-tryptamine reaction (cf. above). Thus, with the type of GA vapour treatment used (100°C , 40 min) only 8% were transformed into the 2-carboxymethyl derivative. Although the GA-tryptamine reaction was performed during longer time, unreacted amine constituted the main part (about 67%) in contrast to what was found in

the GA-DA reaction, whereas only about 15% were found as the tetrahydro derivative. This suggests that the Pictet-Spengler cyclization proceeds more efficiently with DA than with tryptamine, which could be explained by less activation at the point of ring closure on the tryptamine molecule. Interestingly, the second step of fluorophore formation seems to be more efficient with tryptamine than with DA (Papers II and IV). Thus, in the tryptamine reaction about 55% of the total radioactivity recovered with the cyclized products were found in association with strongly fluorescent dihydro- β -carboline. The corresponding figure for dihydroisoquinolines in the GA-DA reaction was only 10%. It should be remembered, however, that the reactions were not performed identically. One possible explanation to the difference would be that the intramolecular acid catalysis of the formation of strongly fluorescent 2-carboxymethyl derivatives (exerted by the 1-COOH group on the tetrahydro- β -carboline and tetrahydroisoquinoline molecules) proceeds more efficiently in the GA-tryptamine reaction. This catalysis was proposed to be responsible for the higher yield of fluorophores from tryptamine in the GA reaction as compared with that obtained in the FA reaction (Paper II); however in the reactions with DA (Paper IV) the fluorophore yields were similar after reaction with GA or FA, which also suggests that the intramolecular acid catalysis might be of less importance in the reaction with DA as compared with that of tryptamine.

c. Fluorophore formation from biogenic amines in the histochemical formaldehyde reaction (Papers II, III, and IV)

From the analyses in Paper III it was concluded that the mechanisms of fluorophore formation, which were analyzed for tryptamine and 5-hydroxytryptamine, most probably are as follows: The reactions between tryptamines and FA have previously been studied by Corrodi and Jonsson (1965 a) and in agreement with their observations it was found that in the first step of the reaction the indolamines react with FA to form low fluorescent 1,2,3,4-tetrahydro- β -carboline. In a subsequent step, these products are converted to fluorophores in either of two ways: through autoxidation to 3,4-dihydro- β -carboline (cf. Corrodi and Jonsson, 1965 a), or through a second, acid-catalyzed reaction with FA yielding 2-methyl-3,4-dihydro- β -carbolinium compounds. Experiments with radioactive tryptamine indicated that the two alternative fluorophore-forming pathways are of fairly equal importance. The FA-induced and acid-catalyzed reaction, which was not previously known, has several interesting properties and implications. First of all, this reaction pathway offers an explanation to the observations of Falck et al. (1962) and Corrodi and Hillarp (1964) that the formation of fluorophores from the initially formed

tetrahydro derivatives is promoted by FA and catalyzed by certain amino acids, peptides or proteins. It is thus likely that amino acids act as acid catalysts of the reaction of FA with the initially formed tetrahydro- β -carboline or tetrahydroisoquinolines, yielding the strongly fluorescent 2-methyl dihydro derivatives. Second, the quantum efficiency of the fluorophores was found to differ considerably, the 2-methyl-substituted dihydro- β -carbolinium compound showing a fluorescence intensity 2-3 times higher than the unsubstituted dihydro- β -carboline when compared on equimolar basis (Paper II). This is interesting because it points to a difference in the fluorescence yield between the two fluorophore forming pathways, the acid-catalyzed reaction of the tetrahydro- β -carboline molecule with FA leading to the formation of the fluorophore with the highest quantum efficiency, the 2-methyl derivative. This suggests new, interesting possibilities of increasing the yield of fluorescence in the Falck-Hillarp method, i.e., by directing the reactions towards the formation of the 2-methyl-substituted fluorophores.

The fluorophore yield in the FA reaction was investigated using ^{14}C -labelled DA and tryptamine (Papers II and IV). After FA vapour treatment alone the yield of strongly fluorescent dihydro derivatives was low both in the DA and the tryptamine reaction, 10% and 5% respectively. However, whereas unreacted tryptamine constituted about 75% after FA treatment, almost no DA was found, the tetrahydroisoquinoline derivative being the main reaction product, constituting about 80%. This points to a similar difference in the efficiency of the initial step in the fluorophore formation, the Pictet-Spengler cyclization, as that found in the GA reaction. Also similar to the GA reaction, FA vapour treatment of FA containing models induced an almost complete transformation of DA to strongly fluorescent dihydroisoquinolines (Paper IV).

III METHODOLOGY

a. Tissue preparation (Papers II, V, and VI)

As the GA molecule has a very limited ability to penetrate into tissues, the GA treatment has to be performed on tissue sections or on thin whole mount preparations. The following modes of application have been tested:

a. Freeze-dried specimens (Papers II and V). The tissue pieces are freeze-dried, embedded in paraffin in vacuo, and sectioned, as described by Björklund et al. (1972 b). The sections are then gently deparaffinized and reacted with GA vapour. The GA vapour treatment can be performed in two ways: 1. The specimens are put in a 1-litre closed vessel containing 800 mg GA monohydrate. The vessel is then placed in an oven at $+100^{\circ}\text{C}$ for 30–40 min. 2. The specimens are exposed to 100–500 torr hot GA vapour at $+100^{\circ}\text{C}$ for 1–10 min as described below.

This type of tissue preparation has been found suitable for peripheral tissues (e.g. pituitary gland, thyroid gland, gastrointestinal tract, skin, pancreas, submaxillary gland; see Paper V and Axelsson et al., 1973). Sections of freeze-dried central nervous tissue, on the other hand, are not useful as the deparaffinization in xylene induces a prominent shrinkage of the tissue structures and the fluorescence yield in monoamine containing structures is quite low.

b. Vibratome sections. This type of application has primarily been devised for the visualization of central CA neurons (Paper VI; Lindvall et al., 1973 a, b). Briefly, the procedure is as follows (for a detailed description of the methodology, see paper VI): Under general barbiturate anesthesia the animals are perfused with an ice-cold Krebs-Ringer bicarbonate buffer containing 2% GA (pH 7.0). Non-perfused tissue or tissue from rats perfused with buffer alone can also be used. The tissue piece is rapidly taken out and then sectioned (about 30 μ thick sections) on a Vibratome instrument. During the sectioning procedure, the tissue piece is immersed in oxygenated, pure Krebs-Ringer bicarbonate buffer (pH 7.0) kept at a low temperature ($0 - +5^{\circ}\text{C}$) by metal bars, cooled to a very low temperature by a dry ice-ethanol mixture. The sections are then transferred with a glass-rod to an ice-cold 2% GA solution (pH 7.0, prepared as above). After the immersion, which should be continued for about 3–5 min, the sections

are transferred to microscope slides. The sections are dried in a two-step procedure: First, the slides are put under the warm air-stream from a hair-dryer for about 15 min. Second, the glasses are kept overnight in vacuo at room temperature, in a dessicator containing fresh phosphorous pentoxide. The sections can be reacted in three different ways: a. GA vapour treatment. 2 g GA monohydrate is heated at $+100^{\circ}\text{C}$ for at least 1 hr in a closed vessel connected to the reaction vessel, which is placed together with the GA vessel in the oven. After evacuation of the reaction vessel with a vacuum pump, hot GA-saturated air (from the GA vessel) is introduced into the hot reaction vessel – containing the tissue specimens – to a partial pressure of 300 torr (mm Hg). Hot air is then let into the reaction vessel to atmospheric pressure. Thus the temperature is approximately $+100^{\circ}\text{C}$ throughout the reaction, which is continued for 2 min. b. Reaction through heating. The sections are placed in an oven at $+100^{\circ}\text{C}$ for 6 min. c. Rapid procedure. The sections are analysed directly after the first step of the drying procedure, i.e. after 15 min under the hair dryer. In this way sections are available for microscopy within half an hour after dissection.

c. Whole mount preparations. In this application, the thin tissues from non-perfused or GA-perfused animals are immersed in the 2% buffered GA solution (pH 7) for 3–5 min. The specimens are then spread out on microscope slides as whole mounts, dried, and reacted with GA vapour or heated as described above.

b. Uptake of catecholamines in Vibratome sections of central nervous tissue (Paper VI)

In these experiments the usefulness of Vibratome sections for studies on the uptake of CAs into CA axons was investigated. The experimental design of Hamberger (1967) was essentially followed. Accordingly, sections of brain tissue from rats treated with reserpine plus nialamide were incubated in DA, with or without desipramine present, and they were then processed according to the GA technique (Paper VI).

With all DA concentrations tested ($5 \times 10^{-7} \text{ M}$ – $5 \times 10^{-5} \text{ M}$) the uptake – as revealed by the intensity of the GA-induced fluorescence – was markedly higher in presumed DA axons, a finding that is in agreement with previous biochemical and fluorescence histochemical findings (Hamberger, 1967; Snyder and Coyle, 1969). Thus, already at the lowest concentration, $5 \times 10^{-7} \text{ M}$, DA terminals in, e.g., the nucleus caudatus-putamen exhibited a strong fluorescence, whereas in presumed NA terminal areas in, e.g., hypothalamus and the sensory-motor cortex only some few fibres had regained a faint fluorescence. With increasing concentration there was a progressive increase in the fluorescence of both DA and NA fibre systems. Significant extraneuronal

DA uptake was detected as a rise in the general background fluorescence of the section after incubation in 10^{-5} – 5×10^{-5} M DA.

According to Hamberger's (1967) observations no uptake of DA into NA structures is demonstrable fluorescence histochemically with the Falck-Hillarp technique when the DA incubation (10^{-6} M, 20 min) is performed in the presence of 10^{-5} M desipramine. This was confirmed with the GA method in the incubated Vibratome sections from, e.g., hypothalamus and cerebral cortex. In contrast, the DA uptake into the DA fibres in the caudate nucleus was seemingly unaffected by desipramine, and here, a distinct, intense fluorescence was obtained in the dense DA terminal network. An unaffected DA uptake was also noted in the system of delicate fibres in the anterior limbic cortex (Paper VI, Lindvall et al., 1974), which have been proposed to be projections from DA cells in the substantia nigra (Björklund et al., 1974). These several observations strongly support the idea that Vibratome sections (from reserpine and nialamide treated animals) incubated in a CA in the presence of desipramine can be used for a direct differentiation between DA and NA neurons in the GA-treated sections, the NA neurons being non-fluorescent after this procedure.

c. Cytofluorometric analysis (Papers IV, V, and VI)

For the characterization and identification of intracellular monoamines with the GA method a knowledge of the fluorescence yields of a number of biogenic amines and related compounds in the GA reaction, and the spectral characteristics of their GA induced fluorophores is of primary importance. With authentic amines and related compounds enclosed in histochemical models these properties were investigated after the three different types of GA reaction used on tissue (see above).

a. Fluorescence yields. After reaction through heating of GA-containing specimens (simulating the conditions of the perfusion-immersion procedure described above; Papers IV and VI), only DOPA, DA and NA gave strong fluorescence. The fluorescence induced from the CAs was about 5-6 times higher than that obtained in the standard FA reaction. No or very weak fluorescence was obtained from adrenaline, from methoxylated PEAs, and from all indolamines tested.

GA vapour treatment of GA-containing models (Papers IV and VI) involved a dramatic increase in the fluorescence yields from the indolamines and methoxylated PEAs tested, and a moderate increase in the yields from the primary CAs and DOPA. Strong fluorescence was induced not only from primary and secondary indolamines but also from the tertiary indolamine (N,N-dimethyltryptamine) and the N-acetylated

indolamines (N-acetyl-5-hydroxytryptamine and melatonin). The fluorescence intensities obtained from the primary and secondary indolamines after the GA vapour treatment were several times higher than that obtained in the standard FA reaction. It was particularly interesting that the N-acetylated and the tertiary indolamines, which give no observable fluorescence upon FA treatment, showed strong fluorescence after GA treatment (see above), the fluorescence yield from melatonin and from N,N-dimethyltryptamine being of the same order of magnitude as that obtained from NA in the FA method. No or only very weak fluorescence was induced from PEAs lacking a cyclization-promoting hydroxyl-group in 3-position (see Corrodi and Jonsson, 1967), phenylethylamine, *p*-tyramine and *p*-octopamine.

With GA vapour treatment alone (i.e. the conditions of the procedure omitting the GA-perfusion and immersion steps; Papers IV, V, and VI, and Axelsson *et al.*, 1973) the selectivity of the reaction was similar to that after GA vapour treatment of GA-containing specimens but the sensitivity for the biogenic amines was lower. However, also with this treatment, the fluorescence yields from the PEAs and indolamines were in most cases several times higher than in the standard FA reaction.

As shown in Paper V, GA vapour treatment induces strong fluorescence from certain tryptophan- or dopa-containing peptides. The fluorescence yields from the NH₂-terminal tryptophan and dopa peptides were considerably higher than those obtained in the FA-ozone and the standard FA reaction, respectively. Also COOH-terminal tryptophan peptides became strongly fluorescent after GA vapour treatment with fluorescence yields similar to that of DA in the Falck-Hillarp method. Differences were recorded between peptides with NH₂- or COOH-terminal tryptophan with respect to their fluorescence yields under different reaction conditions (reaction time, GA concentration in the reaction vessel) and the spectral characteristics of the fluorophores (see below). This offers possibilities for differentiation between the two types of peptides.

b. Spectral characteristics (Papers II, IV, V, and VI). After reaction of GA-containing specimens through heating, the fluorophores of DA, NA, and DOPA – being the only compounds yielding strong fluorescence after this treatment – showed indistinguishable spectra, with excitation and emission maxima at 415 and 475–480 nm. The fluorophores formed from these 3-hydroxylated PEAs (cf. Paper IV) exhibit a pH-dependent tautomerism similar to that of the FA-induced CA fluorophores (Corrodi and Jonsson, 1965 b; Jonsson, 1966; Björklund *et al.*, 1968 a, 1972 a; Paper IV). Thus, at neutral pH – as in the GA-containing specimens, after heating alone – the

fluorophores are in their neutral, quinoidal form (main excitation peak at 415 nm); and at acid pH, such as obtains after GA vapour treatment (see below), they are in their acid, non-quinoidal form (main excitation peak at 370–380 nm). The CA and DOPA fluorophores can also be converted into their acid, non-quinoidal form by exposure to HCl vapour, and furthermore, this treatment provides a possibility for the spectral differentiation between NA on one hand and DA and DOPA on the other (Paper IV), according to the principles previously described by Corrodi and Jonsson (1965 b) and Björklund *et al.* (1968 a, 1972 a) for the FA-induced CA fluorophores. Thus, after about 5 sec of HCl treatment, there is a primary shift of the main excitation peak of all the CA and DOPA fluorophores from 415 nm to 370–380 nm. Upon prolonged treatment there is a further shift of the excitation peak of the NA fluorophore down to 330 nm, whereas those of the DA and DOPA fluorophores remain unchanged at 370–380 nm.

After GA vapour treatment a greater number of biogenic monoamines give strong fluorescence, and on the basis of their emission peak maxima they can be separated into four groups: Group A (emission maxima at 430 nm) comprises N,N-dimethyltryptamine and bufotenin. Group B (emission maxima at 460–485 nm) includes normetanephrine, adrenaline, DOPA, DA, NA, 3-methoxytyramine, N-acetyl-5-hydroxytryptamine and N-methyltryptamine. Group C (emission maxima at 495–500 nm) includes tryptophan, tryptamine, N-methyl-5-hydroxytryptamine and melatonin. Group D (emission maxima at 520–530 nm) comprises 5-hydroxytryptophan, 5-hydroxytryptamine and 5-methoxytryptamine.

Within Group B, the adrenaline and normetanephrine fluorophores (Group B1) can be differentiated from those of the other compounds (Group B2) on the basis of their excitation spectra, which differ markedly directly after the gaseous GA treatment (Paper VI). The primary CAs and DOPA can be distinguished from the other amines in Group B2 because they exhibit the characteristic, pH-dependent shift in their excitation spectra (see above). After GA vapour treatment, the CA fluorophores are primarily in their acid, non-quinoidal form, exhibiting a main excitation peak at 370–380 nm. A short exposure of the sections to ammonia vapour at room temperature will transform them into the neutral, quinoidal state, having a peak maximum (415 nm) that is clearly distinguishable from those of the other compounds in Group B2. Moreover, when GA-containing specimens are reacted through heating, only the primary CAs and DOPA give a strong fluorescence. As pointed out above, the GA-induced fluorophores from DA and NA can, at least in models, be differentiated after HCl treatment.

The excitation and emission spectra of the GA-induced fluorophores of the

different NH_2 -terminal tryptophan peptides are rather similar (Paper V), and show only minor differences from those of the tryptamine and tryptophan fluorophores (see Group C, above). At low to moderate concentrations, the spectra of the COOH-terminal tryptophan peptides are considerably different (spectral characteristics as in Group A, above).

d. Microspectrofluorometric differentiation between dopamine and DOPA (Paper IV)

The fluorophores formed from DA and DOPA in the FA or GA reaction cannot be differentiated on the basis of their spectral characteristics (see, *e.g.*, Jonsson, 1967; Paper VI). The studies described in Paper IV showed that the excitation and emission spectra of the DA and DOPA fluorophores formed in a combined FA and GA reaction differ considerably. The fluorophore formation was proposed to proceed as follows: In a first step (the FA reaction), the tetrahydroisoquinoline and the tetrahydroisoquinoline-3-carboxylic acid, respectively, can be expected to be formed in a Pictet-Spengler type cyclization. In a second step (the GA reaction), the fluorophore formation could proceed in two different ways (*cf.* Svensson *et al.*, 1974): a) the tetrahydroisoquinoline-3-carboxylic acid reacts with a GA molecule yielding the 2-carboxymethyl derivative, which reacts with another GA molecule yielding the fully aromatic isoquinoline, the 2,4-dicarboxymethyl-isoquinolinium compound; b) the tetrahydroisoquinoline also reacts with a GA molecule under the formation of the 2-carboxymethyl derivative. However, no further reaction with GA at the 4-carbon takes place with this molecule. As a result, the fluorophores formed from DA and DOPA in these reactions will have marked structural differences, which are reflected in their spectral properties. The different behaviour of DA and DOPA in the combined FA and GA reaction is most interesting and indicates a possibility for microspectrofluorometric differentiation between these substances at the cellular level.

e. Specificity

On the basis of the knowledge of the GA-monoamine reactions (see Papers II and IV) and the identity of the fluorescent products, the histochemical tests of specificity in the GA method should aim at a characterization of a) the conditions of fluorescence development, and b) the nature of the fluorophore.

a. Conditions of fluorescence development. It is a basic requirement that the fluorescence is GA-induced, thus not occurring in tissue not exposed to GA. With GA-

-containing specimens, a fluorescence due to DA, NA, or DOPA will develop during the drying procedure of the sections and it will become stronger after either the subsequent heating or the subsequent GA vapour treatment. Other fluorogenic monoamines will become significantly fluorescent only after a GA vapour treatment, and it is clear that the fluorescence yield from these amines will be much dependent on the conditions of the GA vapour treatment and perhaps of other steps in the procedure as well. Thus, the GA concentration in the reaction vessel will strongly influence the fluorescence yield primarily from the N-acetylated and tertiary indolamines, the 3-methoxylated CAs, and COOH-terminal tryptophan peptides, whereas reaction time will influence the fluorescence yield primarily from the secondary PEAs, COOH-terminal tryptophan peptides, and the 3-methoxylated CAs. It should be noted in this context, that our experience from studies on the rat CNS is that only DA and NA will yield significant fluorescence in the brain sections when the GA method is performed on GA-containing specimens (see Paper VI and above).

b. Characterization of the fluorophore. This is best done with microspectrofluorometric analysis as described above. However, in the fluorescence microscope, both the colour of the GA-induced fluorescence, and the intensity of the fluorescence at different activation wavelengths, should be used as general screening tests. The pH-dependent tautomeric shift in the excitation spectra of the CA fluorophores is of special value for their characterization, because this shift can be observed directly in the fluorescence microscope (see Paper VI).

In conjunction with the histochemical tests, the use of pharmacological tests, such as have been used extensively in the applications of the Falck-Hillarp method (see Corrodi and Jonsson, 1967; Björklund et al., 1972 b), can be recommended. Such tests involve for example disappearance of the fluorescence after the administration of drugs that reduce the amine stores (e.g. the releasing agents reserpine and α -methyl-m-tyrosine or the synthesis inhibitors α -methyl-p-tyrosine and p-chlorophenylalanine), or an increased fluorescence after administration of drugs that cause an accumulation of the amines (e.g. monoamine oxidase inhibitors).

IV NEUROANATOMICAL APPLICATION

a. The fluorescence microscopical picture of central CA neurons (Paper VI)

With the GA method applied to Vibratome sections (see above), DA and NA containing neurons become strongly fluorescent. Their blueish-green, UV-stable fluorescence differs from the UV-sensitive, brownish-yellow, weak and variable fluorescence induced in indolamine-containing neurons. In fact, in its present design, the GA method appears to be essentially selective for DA- and NA-containing structures.

In the GA treated sections, as in Vibratome sections processed according to the FA technique (Hökfelt and Ljungdahl, 1972), the morphology of the CA-containing cell bodies is of variable quality. Thus, the fluorescence often covers the nucleus, and the cell bodies sometimes look distorted and have a diffuse outline. The cellular fluorescence is, however, in many cases considerably stronger than that observed in freeze-dried, FA treated specimens.

Interestingly, a GA-induced fluorescence is also observed in the dendrites of many CA cells. Most often only the proximal parts of the dendrites are clearly fluorescent. A notable exception to this has been discovered in the substantia nigra, where the presumed dendrites of the DA-containing cells in the pars compacta (group A9 of Dahlström and Fuxe, 1964) project into the pars reticulata, giving rise to a loose network with small, mostly spindle-shaped varicosities quite regularly spaced along the fibres (Björklund and Lindvall, 1974).

In almost all CA neuron systems analysed not only the terminal parts (see below) but also the entire preterminal axon becomes fluorescent, although the fluorescence in the initial segment of the axon is often rather weak. Thus, the main CA-bundles – previously described with the Falck-Hillarp method in conjunction with lesions – can be followed from their cell bodies of origin up to the terminal areas in intact, untreated adult animals. Moreover, due to the lack of diffusion, the detailed fluorescence morphology of the individual fibres can be observed. This has proved to be of great value in neuroanatomical work (see Papers VII and VIII), since axons of the same origin have been found to exhibit characteristic morphological features, and in many cases the axonal morphology of different systems is clearly different and

distinguishable in the fluorescence microscope. This can be used to trace individual systems of axons also in regions and bundles where different systems occur intermingled with each other.

CA-containing terminal and paraterminal axonal ramifications, demonstrable with the Falck-Hillarp technique (see Fuxe, 1965 a, b) are also observed in the GA specimens. The smooth, intervarticose segments of the terminal fibres are most often clearly visible. The fluorescence picture is extremely distinct and without any signs of diffusion, and consequently the morphology of the individual fibre can be studied. This is particularly evident, e.g., in the caudate nucleus, which has a diffuse fluorescence in specimens processed according to the Falck-Hillarp FA method.

Of particular interest for neuroanatomical tracing is the finding that terminal fibres of the same origin, but localized in different brain areas, have – at least in some cases – a very similar fluorescence appearance. A classification of terminals, on the basis of their fluorescence appearance in the GA method, thus seems possible and should provide a very useful tool for the identification of projections from one and the same group of cells in different brain regions, as well as for the distinction between different terminal systems in one and the same brain region (cf. Papers VII and VIII).

b. The organization of the ascending CA neuron systems in the rat brain (Paper VII)

Owing to the low amine concentration in the non-terminal axon, visualization of this part of the CA neuron with the Falck-Hillarp method is usually not possible in the CNS of intact and untreated adult animals. To obtain this, the production of lesions of the axons is required in order to increase the intra-axonal amine concentration, which can be achieved either mechanically (Dahlström and Fuxe, 1965), or chemically by means of 6-hydroxydopamine (Ungerstedt, 1971) or 6-hydroxydopa (Jacobowitz and Kostrzewa, 1971; Sachs and Jonsson, 1972). In young animals, on the other hand, the transmitter content of the preterminal axons is higher and at least some axonal systems can for this reason be demonstrated already in the intact animal (Maeda and Dresse, 1968; Olson and Fuxe, 1971).

The NA and DA containing cell groups in the lower brain stem are known to give rise to widespread ascending projections to many diencephalic and telencephalic regions. Although much information has been gained about the projections of the CA neuron systems, above all through the extensive work of Fuxe, Ungerstedt and collaborators (Andén et al., 1966; Dahlström and Fuxe, 1964; Fuxe, 1965 b; Olson and Fuxe, 1971, 1972; Ungerstedt, 1971) and of Maeda and collaborators (Maeda et al.,

1973; Maeda and Shimizu, 1972), the knowledge of the organization of the CA systems is still far from complete, which can, at least partly, be referred to the difficulties inherent in the methodology employed (cf. above).

In Paper VII the GA method was applied to obtain a more detailed picture of the organization of the several CA neuron systems and their modes of projection in the forebrain. It was found that the rostrally projecting fibres from the mesencephalic, pontine, and medullary CA cell groups are, generally speaking, primarily associated with four major conduction pathways (for a detailed description, see Paper VII): the central tegmental tract, the periventricular system, the medial forebrain bundle, and the nigrostriatal pathway. To a remarkable extent the CA pathways follow, along or reciprocal to, non-adrenergic fibre tracts well known from classical neuroanatomy.

The CA fibres in the central tegmental tract could be traced along the myelinated tegmental fascicles from the medulla oblongata up to the caudal diencephalon. The ascending medullary CA axons assemble largely into a bundle situated just ventro-lateral to the hypoglossal nucleus and ventral to the dorsal vagal nucleus. Going rostrally this medullary bundle passes just ventral to the genu of the facial nerve. In the pons the number of CA axons and the size of the CA fibre system in the central tegmental tract increase considerably owing to the inflow of ascending pontine fibres from at least three major sources: from the so-called A5 cell group, from the subcoeruleus cell group, and from the locus coeruleus cell group. The most substantial rostral projection from the locus coeruleus is confined to a separate bundle, the dorsal tegmental bundle, running in the dorsomedial part of the CA fibre system of the central tegmental tract. This bundle, which is identical to the so-called dorsal bundle of Ungerstedt (1971), ascends through the zona incerta and along the medial forebrain bundle to give fibres to the thalamus, neocortex, and hippocampus, and to a lesser extent also to other areas (for a detailed description of the projection routes of this bundle, see Paper VII). Other pontine and medullary CA fibres in the central tegmental tract pass in a complex manner through the so-called tegmental radiations in the mesencephalon to contribute to the ascending CA fibre system in the medial forebrain bundle. Part of the central tegmental tract fibres run rostrally into the supraoptic decussations and through the zona incerta, the H₂-field of Forel and the internal capsule towards the ansa lenticularis, the neostriatum, and possibly also the overlying cortex.

The periventricular system was demonstrated along the periventricular and periaqueductal gray, from the medulla oblongata up to the rostral diencephalon and the septal region. A distinction was made between a dorsal system of fibres extending along the dorsal longitudinal fasciculus, and a ventral system extending along the peri-

ventricular gray of the hypothalamus. A characteristic feature of the dorsal periventricular system is that many of its cell bodies of origin are distributed diffusely along its extent through pons, mesencephalon, and posterior thalamus. In addition, the locus coeruleus CA cell bodies were seen to contribute significantly to this fibre system. The ventral periventricular system could be traced from the rostral mesencephalon through the supramamillary region and the posterior hypothalamic area into the medial part of the dorsomedial hypothalamic nucleus. Here the system probably receives fibres from the dorsal periventricular bundle, and possibly also from the medial forebrain bundle, to form a broad ascending hypothalamic CA fibre system dispersed on the lateral aspect of the periventricular nucleus.

The CA fibre systems of the medial forebrain bundle assemble, at the meso-diencephalic junction, from the rostral outflow of the so-called tegmental CA radiations (comprising medullary and pontine fibres of the central tegmental tract), from the mesencephalic CA cell groups, and to a minor extent probably also from the dorsal tegmental bundle, the dorsal periventricular system and the mamillary peduncle (which also carries CA fibres). Further rostrally, in the middle hypothalamus, the dorsal tegmental bundle joins the system. As revealed with the GA method the medial forebrain bundle system is highly heterogeneous with respect to its CA fibres, and it comprises both NA- and DA-containing systems whose projection routes have been described in some detail. Previously unknown, presumed DA-containing fibre systems innervating the frontal and anterior limbic cortices were detected in the GA treated material. Also the dopaminergic nigrostriatal pathway and mesolimbic system were well traceable throughout their extent, from the cell bodies of origin in the rostral mesencephalon up to their terminal areas. The topography and courses of these fibre systems are described in Paper VII.

c. The adrenergic innervation of the rat thalamus (Paper VIII)

In specimens processed according to the standard Falck-Hillarp method the only areas of the thalamus, epithalamus, metathalamus, and subthalamus that appear to have dense CA innervations are the anteroventral and paraventricular nuclei and the dorsal nucleus of the lateral geniculate body (Fuxe, 1965 b; Fuxe et al., 1969). Thus, the CA fibre supply to most areas in the thalamus appears insignificant in comparison to that of many other brain regions (see Fuxe, 1965 b) and for this reason, effects at the thalamic level of manipulations of the adrenergic systems by lesions or pharmacological agents have seldom been considered.

With the GA method more abundant innervations were observed in nuclei where CA-containing fibres previously have been detected in low or very low densities, e.g., in the reticular and the ventral thalamic nuclei and the medial geniculate body. Rich supplies of adrenergic fibres were demonstrated also in regions that have been reported to lack adrenergic innervation (Fuxe, 1965 b), e.g., in the anteromedial, gelatinous, rhomboidal and parafascicular nuclei of the thalamus, in the lateral habenular nucleus, and in the zona incerta of the subthalamus. In combination with stereotaxic lesions the following CA systems to the thalamus could be established:

1. The locus coeruleus system. Most of these axons ascend in the dorsal tegmental bundle (cf. above and Paper VII), from which branches were traced along several routes, giving rise to extensive terminal systems in many thalamic, metathalamic and pretectal areas, most notably the anterior, ventral and lateral nuclear complexes, and the medial and lateral geniculate bodies.

2. The dorsal periventricular bundle (cf. above and paper VII). This system originates in cell bodies (defined as the A11 cell group) in the dorsal raphe region, the central gray of the mesencephalon, and in the periventricular gray of the caudal thalamus. The axons ascend within the dorsal longitudinal fasciculus and give rise to a thalamic and hypothalamic periventricular system, projecting to medial and midline thalamic, epithalamic and pretectal regions.

3. Part of the terminals in the paraventricular thalamic nucleus was identified with a non-locus projection from cell bodies in the pontine or medullary reticular formation.

4. A system of delicate, probably DA-containing axons was revealed in the caudal thalamus, the zona incerta and the dorsal and anterior hypothalamus. This system probably originates in the DA cell bodies of the diencephalic A11 and A13 cell groups, forming a hitherto unknown intradiencephalic dopaminergic system.

V CONCLUSIONS

A. Aldehydes, ketones, α -keto acids and carboxylic acids were found to induce significant visible fluorescence from CAs and indolamines under histochemical reaction conditions. FA and GA seemed to be the reagents most suited for histochemical application, combining high reactivity with good selectivity. Interestingly, some reagents formed fluorophores with N-acetylated indolamines, *p*-tyramine and histamine, compounds that cannot be demonstrated with sufficient sensitivity with available techniques.

B. GA was found to be more sensitive than FA as reagent for the visualization of CAs, methoxylated CAs, indolamines, and NH_2 -terminal tryptophan and dopa peptides, and to induce strong fluorescence from compounds that cannot be visualized with the Falck-Hillarp technique, *e.g.*, N-acetylated and tertiary indolamines, and COOH -terminal tryptophan peptides.

C. The reactions between GA and primary PEAs and indolamines were investigated in detail for DA and tryptamine, respectively. The fluorophore formation was shown to proceed in two principal reaction steps: The PEA or indolyethylamine reacts with GA in a Pictet-Spengler cyclization yielding the 1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid or 1,2,3,4-tetrahydro- β -carboline-1-carboxylic acid, respectively, via a Schiff's base. These very weakly fluorescent compounds are transformed to strongly fluorescent dihydroisoquinolines and dihydro- β -carbolines mainly via an intramolecularly catalyzed reaction with a second GA molecule yielding the corresponding 2-carboxymethyl derivatives.

D. The mechanisms of fluorophore formation for primary indolamines in the FA reaction were investigated and concluded to be as follows: In the first step of the reaction the indolamines react with FA to form low fluorescent 1,2,3,4-tetrahydro- β -carbolines. In a subsequent step, these products are converted to fluorophores in either of two ways: through autoxidation to 3,4-dihydro- β -carbolines, or through a second, acid-catalyzed reaction with FA yielding 2-methyl-3,4-dihydro- β -carbolinium compounds. The latter of these fluorophore-forming reactions was not previously known; it points to interesting possibilities of increasing the sensitivity of the Falck-Hillarp method.

E. By means of radioactively labelled DA and tryptamine it was shown that the fluorophore

yields in the standard FA and GA reactions are far from optimum. This indicated possibilities of increasing the sensitivity of the techniques by directing the reactions towards quantitative yields of the fluorophore, with the highest quantum efficiency. In fact, when performed under conditions simulating the perfusion-immersion procedure used on tissue, DA was almost quantitatively transformed into strongly fluorescent dihydroisoquinolines both in the FA and GA reaction.

F. The GA method was applied to tissue and it was shown that, when carried out on Vibratome sections, this technique demonstrates central CA-containing neurons with an extraordinary sensitivity and richness in details. In freeze-dried tissue the GA method demonstrated intracellular monoamines such as DA, NA, 5-hydroxytryptamine and a NH_2 -terminal tryptophan peptide with high sensitivity.

G. The possibilities for microspectrofluorometric identification of and differentiation between various fluorogenic monoamines in the GA method were investigated in detail. Of particular interest was the finding that the fluorophores formed from DOPA and DA in a combined FA and GA reaction showed different spectral characteristics, indicating a possibility for a direct histochemical differentiation between these closely related compounds by microspectrofluorometry.

H. With the GA method the detailed organization of the ascending CA neuron systems in the intact, adult rat brain was investigated. The rostrally projecting fibres from the mesencephalic, pontine, and medullary CA cell groups were found to be primarily associated with four major conduction pathways: the central tegmental tract, the periventricular system, the medial forebrain bundle, and the nigrostriatal pathway. The identity, composition, course, and primary branching patterns of these rostrally projecting CA fibre tracts were described.

I. Many areas of the thalamus, epithalamus, metathalamus, and subthalamus that appear sparsely innervated by CA fibres in specimens processed according to the standard Falck-Hillarp method, were found to be richly supplied with such fibres in the GA-treated specimens. With the GA method the density and fibre pattern of the adrenergic innervation of the various nuclei in the thalamus and adjoining regions were investigated and the origins of the CA innervations studied by the use of lesions.

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REFERENCES

- Andén, N.-E., Dahlström, A., Fuxe, K., Larsson, K., Olson, L. and Ungerstedt, U.: Ascending monoamine neurons to the telencephalon and diencephalon. Acta physiol. scand. 1966, 67, 313-326.
- Angelakos, E.T. and King, M.P.: Demonstration of nerve terminals containing adrenaline by a new histochemical technique. Nature (Lond.) 1967, 213, 391-393.
- Axelsson, S., Björklund, A., Falck, B., Lindvall, O. and Svensson, L.Å.: Glyoxylic acid condensation: a new fluorescence method for the histochemical demonstration of biogenic monoamines. Acta physiol. scand. 1973, 87, 57-62.
- Björklund, A., Ehinger, B. and Falck, B.: A method for differentiating dopamine from noradrenaline in tissue sections by microspectrofluorometry. J. Histochem. Cytochem. 1968 a, 16, 263-270.
- Björklund, A., Ehinger, B. and Falck, B.: Analysis of fluorescence excitation peak ratios for the cellular identification of noradrenaline, dopamine, or their mixtures. J. Histochem. Cytochem. 1972 a, 20, 56-64.
- Björklund, A., Falck, B. and Håkanson, R.: Histochemical demonstration of tryptamine. Properties of the formaldehyde-induced fluorophores of tryptamine and related indole compounds in models. Acta physiol. scand. 1968 b, suppl. 318.
- Björklund, A., Falck, B. and Owman, Ch.: Fluorescence microscopic and microspectrofluorometric techniques for the cellular localization and characterization of biogenic amines. In: Methods of investigative and diagnostic endocrinology (ed.: S.A. Berson), vol. 1: The thyroid and biogenic amines (eds.: J.E. Rall and I.J. Kopin). Amsterdam: North-Holland Publ. Comp. 1972 b, p. 318-368.
- Björklund, A. and Lindvall, O.: Dopamine-containing dendrites in the pars reticulata of the substantia nigra. To be published 1974.
- Björklund, A., Lindvall, O., Moore, R.Y. and Stenevi, U.: Adrenergic neocortical innervation: organization of dopaminergic systems from the midbrain. To be published 1974.

- Björklund, A., Nobin, A. and Stenevi, U.: Acid catalysis of the formaldehyde condensation reaction for a sensitive histochemical demonstration of tryptamines and 3-methoxylated phenylethylamines. 2. Characterization of amine fluorophores and application to tissues. J. Histochem. Cytochem. 1971, 19, 286-298.
- Björklund, A. and Stenevi, U.: Acid catalysis of the formaldehyde condensation reaction for sensitive histochemical demonstration of tryptamines and 3-methoxylated phenylethylamines. 1. Model experiments. J. Histochem. Cytochem. 1970, 18, 794-802.
- Carlsson, A., Falck, B., Hillarp, N.-Å., Thieme, G. and Torp, A.: A new histochemical method for visualization of tissue catecholamines. Med. Exp. 1961, 4, 123-125.
- Corrodi, H. and Hillarp, N.-Å.: Fluoreszenzmethoden zur histochemischen Sichtbarmachung von Monoaminen. 1. Identifizierung der fluoreszierenden Produkte aus Modellversuchen mit 6,7-Dimethoxyisochinolinderivaten und Formaldehyd. Helv. Chim. Acta 1963, 46, 2425-2430.
- Corrodi, H. and Hillarp, N.-Å.: Fluoreszenzmethoden zur histochemischen Sichtbarmachung von Monoaminen. 2. Identifizierung des fluoreszierenden Produktes aus Dopamin und Formaldehyd. Helv. Chim. Acta 1964, 47, 911-918.
- Corrodi, H. and Jonsson, G.: Fluoreszenzmethoden zur histochemischen Sichtbarmachung von Monoaminen. 5. Identifizierung des fluoreszierenden Produktes aus Modellversuchen mit 5-Methoxytryptamine und Formaldehyd. Acta histochem.(Jena) 1965 a, 22, 247-258.
- Corrodi, H. and Jonsson, G.: Fluorescence methods for the histochemical demonstration of monoamines: 4. Histochemical differentiation between dopamine and norepinephrine in models. J. Histochem. Cytochem. 1965 b, 13, 484-487.
- Corrodi, H. and Jonsson, G.: The formaldehyde fluorescence method for the histochemical demonstration of biogenic monoamines. A review on the methodology. J. Histochem. Cytochem. 1967, 15, 65-78.
- Dahlström, A. and Fuxe, K.: Evidence for the existence of monoamine neurons in the central nervous system. I. Demonstration of monoamines in the cell bodies of brain stem neurons. Acta physiol. scand. 1964, 64, suppl. 232.
- Dahlström, A. and Fuxe, K.: Evidence for the existence of monoamine neurons in the central nervous system. II. Experimentally induced changes in the intraneuronal amine levels of bulbospinal neuron systems. Acta physiol. scand. 1965, 64, suppl. 247.

- Dahlström, A., Häggendal, J. and Hökfelt, T.: The noradrenaline content of the varicosities of sympathetic adrenergic nerve terminalis in the rat. Acta physiol. scand. 1966, 67, 289-294.
- Ehinger, B. and Thunberg, R.: Induction of fluorescence in histamine-containing cells. Exp. Cell Res. 1967, 47, 116-122.
- Falck, B.: Observations on the possibilities of the cellular localization of monoamines by a fluorescence method. Acta physiol. scand. 1962, 56, suppl. 197.
- Falck, B., Hillarp, N.-Å., Thieme, G. and Torp, A.: Fluorescence of catecholamines and related compounds condensed with formaldehyde. J. Histochem. Cytochem. 1962, 10, 348-354.
- Fuxe, K.: Evidence for the existence of monoamine neurons in the central nervous system. III. The monoamine nerve terminal. Z. Zellforsch. 1965 a, 65, 573-596.
- Fuxe, K.: Evidence for the existence of monoamine neurons in the central nervous system. IV. The distribution of monoamine nerve terminals in the central nervous system. Acta physiol. scand. 1965 b, 64, suppl. 247.
- Fuxe, K., Hökfelt, T. and Ungerstedt, U.: Distribution of monoamines in the mammalian central nervous system by histochemical studies. In: Metabolism of amines in the brain (ed.: E. Hooper). London: Mac Millan 1969, p. 10-22.
- Hamberger, B.: Reserpine-resistant uptake of catecholamines in isolated tissues of the rat. Acta physiol. scand. 1967, suppl. 295.
- Håkanson, R. and Owman, Ch.: Concomitant histochemical demonstration of histamine and catecholamines in enterochromaffin-like cells of gastric mucosa. Life Sci. 1967, 6, 759-766.
- Håkanson, R. and Sundler, F.: Formaldehyde condensation. A method for the fluorescence microscopic demonstration of peptides with NH_2 -terminal tryptophan residues. J. Histochem. Cytochem. 1971, 19, 477-482.
- Hökfelt, T. and Ljungdahl, Å.: Modification of the Falck-Hillarp formaldehyde fluorescence method using the Vibratome[®]: simple, rapid and sensitive localization of catecholamines in sections of unfixed or formalin fixed brain tissue. Histochemie 1972, 29, 325-339.
- Jacobowitz, D. and Kostrzewa, R.: Selective action of 6-hydroxydopa on noradrenergic terminals: Mapping of preterminal axons of the brain. Life Sci. 1971, 10, 1329-1342.

- Jonsson, G.: Fluorescence studies on some 6,7-substituted 3,4-dihydroisoquinolines formed from 3-hydroxytyramine (dopamine) and formaldehyde. Acta Chem. Scand. 1966, 20, 2755-2762.
- Jonsson, G.: The formaldehyde fluorescence method for the histochemical demonstration of biogenic monoamines. A methodological study. M.D. Thesis, Stockholm 1967.
- Jonsson, G.: Microfluorimetric studies on the formaldehyde-induced fluorescence of noradrenaline in adrenergic nerves of rat iris. J. Histochem. Cytochem. 1969, 17, 714-723.
- Juhlin, L. and Shelley, W.B.: Detection of histamine by a new fluorescent o-phthalaldehyde stain. J. Histochem. Cytochem. 1966, 14, 525-528.
- Kovács, Ö. and Fodor, G.: Beitrag zur Synthese der Tetrahydroisochinolin-Alkaloide unter physiologischen Bedingungen. Ber. dtsch. chem. Ges. 1951, 84, 795-801.
- Lindvall, O., Björklund, A. and Falck, B.: Glyoxylic acid condensation: A new fluorescence histochemical method for sensitive and detailed tracing of central catecholamine neurons. In: Frontiers in catecholamine research (eds.: E. Usdin and S. Snyder). Oxford: Pergamon Press 1973 a, p. 683-687.
- Lindvall, O., Björklund, A., Hökfelt, T. and Ljungdahl, Å.: Application of the glyoxylic acid method to Vibratome sections for improved visualization of central catecholamine neurons. Histochemie 1973 b, 35, 31-38.
- Lindvall, O., Björklund, A. and Moore, R.Y.: The adrenergic innervation of the neocortex as revealed by the glyoxylic acid fluorescence method. To be published 1974.
- Olson, L. and Fuxe, K.: On the projections from the locus coeruleus noradrenaline neurons: The cerebellar innervation. Brain Res. 1971, 28, 165-171.
- Olson, L. and Fuxe, K.: Further mapping out of central noradrenaline neuron systems: Projections of the subcoeruleus area. Brain Res. 1972, 43, 289-295.
- Maeda, T. and Dresse, A.: Possibilités d'étude du trajet des fibres cérébrales monoaminergiques chez le rat nouveau-né. C.R. Soc. Biol. (Paris) 1968, 162, 1626-1629.
- Maeda, T., Pin, C., Salvert, D., Ligier, M. and Jouvét, M.: Les neurones contenant des catécholamines du tegmentum pontique et leurs voies de projection chez le chat. Brain Res. 1973, 57, 119-152.
- Maeda, T. and Shimizu, N.: Projections ascendantes du locus coeruleus et d'autres neurones aminergiques pontiques au niveau du prosencephale du rat. Brain Res. 1972, 36, 19-36.

- Rost, F.W.D. and Ewen, S.W.B.: New methods for the histochemical demonstration of catecholamines, tryptamines, histamine and other arylethylamines by acid- and aldehyde-induced fluorescence. Histochem. J. 1971, 3, 207-212.
- Sachs, Ch. and Jonsson, G.: Degeneration of central and peripheral noradrenaline neurons produced by 6-hydroxy-DOPA. J. Neurochem. 1972, 19, 1561-1575.
- Snyder, S.H. and Coyle, J.T.: Regional differences in H^3 -norepinephrine and H^3 -dopamine uptake into rat brain homogenates. J. Pharmacol. Exp. Ther. 1969, 165, 78-86.
- Svensson, L.-Å., Björklund, A., and Lindvall, O.: Studies on the reactions between glyoxylic acid and various catecholamines and tetrahydroisoquinolines. To be published 1974.
- Ungerstedt, U.: Stereotaxic mapping of the monoamine pathways in the rat brain. Acta physiol. scand. 1971, suppl. 367.
- Whaley, W.M. and Govindachari, T.R.: The Pictet-Spengler synthesis of tetrahydroisoquinolines and related compounds. In: Organic reactions. Coll. vol. 6 (ed.: R. Adams). New York: John Wiley and Sons, Inc. 1951, p. 151-206.

